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<b>(21) International Application Number:</b> PCT/US99/25359 <b>(22) International Filing Date:</b> 29 October 1999 (29.10.1999) <b>(30) Priority Data:</b> 60/106,491 30 October 1998 (30.10.1998) US <b>(60) Parent Application or Grant</b> NORTHWESTERN UNIVERSITY [/]; (). JONES, Jonathan, C., R. [/]; (). BAKER, Scott, E. [/]; (). CROOK, Wannell, M. ; ().	<b>Published</b>	
<b>(54) Title: METHOD OF INTERFERING WITH CELL PROLIFERATION</b> <b>(54) Titre: METHODE PERMETTANT D'INFLUENCER LA PROLIFERATION CELLULAIRE</b>  <b>(57) Abstract</b> <p>The invention provides a method for interfering with cell proliferation by contacting cells with a molecule which binds to, and inhibits the function of, laminin 5 or 'alpha'3'beta'1 integrins. This method is useful for inhibiting the proliferation of a variety of cell types, particularly tumor cells. Also provided are pharmaceutical compositions comprising a molecule of the invention and a pharmaceutically-acceptable carrier.</p> <b>(57) Abrégé</b> <p>L'invention se rapporte à une méthode permettant d'influencer la prolifération cellulaire et consistant à mettre en contact une molécule qui se lie à la laminine 5 ou aux intégrines 'alpha'3'beta'1 et qu'elle inhibe. Cette méthode permet d'inhiber la prolifération de nombreux types de cellules, notamment les cellules tumorales. L'invention se rapporte également à des compositions pharmaceutiques comportant une molécule telle que celle décrite ci-dessus et un excipient pharmaceutiquement acceptable.</p>		

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<b>(21) International Application Number:</b> PCT/US99/25359 <b>(22) International Filing Date:</b> 29 October 1999 (29.10.99)  <b>(30) Priority Data:</b> 60/106,491 30 October 1998 (30.10.98) US  <b>(71) Applicant:</b> NORTHWESTERN UNIVERSITY [US/US]; 1801 Maple Avenue, Evanston, IL 60201-3135 (US).  <b>(72) Inventors:</b> JONES, Jonathan, C., R.; 1730 N. Wells, Chicago, IL 60614 (US). BAKER, Scott, E.; 720 N. 6th Avenue #305, Tucson, AZ 85705 (US).  <b>(74) Agents:</b> CROOK, Wannell, M. et al.; Sheridan Ross P.C., Suite 1200, 1560 Broadway, Denver, CO 80202-5141 (US).	<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> METHOD OF INTERFERING WITH CELL PROLIFERATION  <b>(57) Abstract</b>  The invention provides a method for interfering with cell proliferation by contacting cells with a molecule which binds to, and inhibits the function of, laminin 5 or $\alpha_3\beta_1$ integrins. This method is useful for inhibiting the proliferation of a variety of cell types, particularly tumor cells. Also provided are pharmaceutical compositions comprising a molecule of the invention and a pharmaceutically-acceptable carrier.		

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**Description**

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## METHOD OF INTERFERING WITH CELL PROLIFERATION

### FIELD OF THE INVENTION

The present invention relates to a method of interfering with cell proliferation by contacting a cell with a molecule that binds to, and inhibits the function of, laminin 5 or  $\alpha_3\beta_1$  integrins.

### BACKGROUND OF THE INVENTION

Although cell proliferation is often desirable, aberrant cell proliferation is often deleterious. Cell proliferation is a vital process which occurs during naturally-occurring physiological processes, such as wound healing, immune responses, bone repair, inflammation and the like. However, uncontrolled cell proliferation can lead to undesirable conditions, such as tumorigenesis. Although chemotherapy agents and/or radiation treatments inhibit tumor growth, these treatments are not entirely satisfactory because the tumor cells often develop drug resistance.

Laminins are heterotrimeric extracellular matrix proteins consisting of three subunits:  $\alpha$ ,  $\beta$  and  $\gamma$ . There are at least five known  $\alpha$  subunits ( $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5$ ), three known  $\beta$  subunits ( $\beta_1, \beta_2, \beta_3$ ) and two known  $\gamma$  ( $\gamma_1, \gamma_2$ ) subunits (Miner et al., *J. Cell. Biol.* 137:685-701, 1997). Laminin 5 (LN5) is an  $\alpha_3\beta_3\gamma_2$  heterotrimer which is typically associated with epithelial cell adhesion and sometimes with hemidesmosome formation. The designation "laminin 5" was coined by Burgeson et al. (*Matrix Biol.* 14:209-211, 1994) to refer to a protein which is secreted into the culture medium by human keratinocytes and enhances keratinocyte attachment (Rousselle et al., *J. Cell Biol.* 114:567-576, 1991; International Publication Nos. WO92/17498 and WO94/0531). A similar protein was also identified by Carter et al. (*Cell* 65:599-619, 1991; International Publication No. WO95/06660) and called epiligrin. This protein is similar to the basement membrane glycoprotein recognized by the GB<sub>3</sub> antibody in human keratinocyte culture medium called nicein (Hsi et al., *Placenta* 8:209-217, 1987). LN5 is also produced by 804G and NBT-II rat bladder carcinoma cells (U.S. Patents Nos. 5,541,106 and 5,422,264). A human epithelial cell line, MCF-10A, produces a LN5 extracellular matrix which also induces hemidesmosome formation. This extracellular matrix protein is described in U.S. Patent No. 5,770,448. U.S. Patents Nos. 5,422,264 and 5,541,106

5 describe the isolation of rat LN5 and its ability to induce adhesion and hemidesmosome  
formation in epithelial cells. The purification of soluble LN5 is described in U.S. Patent  
10 No. 5,760,179. U.S. Patents Nos. 5,510,263 and 5,681,587 disclose the successful  
passaging of fetal and adult islet cells when plated on a rat LN5-coated substrate. U.S.  
5 Patent No. 5,672,361 discloses the growth of pancreatic islet cells on human LN5-coated  
substrates. U.S. Patent No. 5,585,267 discloses the growth of epithelial cells on trans-  
15 epithelial appliances coated with rat LN5. U.S. Patent Application Serial No. 09/145,387  
discloses the use of LN5 for treatment of bone defects. Finally, U.S. Patent Application  
Serial No. 09/232,394 discloses two types of LN5. One contains unprocessed  $\alpha_3$  subunits  
20 and promotes the migration of epithelial cells. The other contains processed  $\alpha_3$  subunits  
and promotes hemidesmosome assembly. The entire contents of U.S. Patents Nos.  
5,422,264, 5,510,263, 5,541,106, 5,585,267, 5,672,361, 5,681,587, 5,760,179, and  
25 5,770,448 and of U.S. Patent Applications Serial Nos. 09/145,387 and 09/232,394 are  
hereby incorporated by reference.

15 LN5 is a matrix component of epithelial tissue basement membranes and plays an  
important role in the initiation and maintenance of epidermal cell anchorage to the  
underlying connective tissue. The interaction of cells with elements of the extracellular  
30 matrix impacts their adherence and motility, as well as protein and gene expression  
(Adams et al., *Develop.* 117:1183-1198, 1993). In intact, normal tissue, epithelial cells  
20 bind to extracellular matrix molecules which are organized into a complex multiprotein  
structure called the basement membrane. The major components of the basement  
membrane include type IV collagen, proteoglycans, and laminin. LN5 plays an important  
role in establishing firm adherence of epithelial cells to the basement membrane since it is  
40 necessary for the assembly and maintenance of stable anchorage devices between epithelial  
cells and hemidesmosomes (Green et al., *FASEB J.* 10:871-880, 1996; Baker et al., *J. Cell*  
25 *Sci.* 109:2509-2520, 1996). LN5 is also expressed at the budding tips of invading tumor  
cell populations, *i.e.* at sites where cancer cells are undergoing cell division but where  
45 there are most likely no hemidesmosomes (Pyke et al., *Am. J. Pathol.* 145:782-791, 1994;  
Pyke et al., *Cancer Res.* 55:4132-4139, 1995).

30 Two cell adhesion receptors (integrins) which bind LN5 have been identified:  $\alpha_3\beta_1$   
50 and  $\alpha_6\beta_4$ . Integrins are heterodimeric cell surface proteins having an  $\alpha$  chain and a  $\beta$  chain

which bind extracellular matrix molecules and are involved in signal transduction upon binding these molecules. A variety of epithelial cells use the  $\alpha_3\beta_1$  integrin to bind LN5 *in vitro* (Carter et al., *Cell* 65:599-610, 1991). Many other cell types also express  $\alpha_3\beta_1$ , including muscle cells and fibroblasts. However, for some cells this interaction appears to be transitory and, both *in vitro* and *in vivo*, cell interaction with LN5 at some point switches to the  $\alpha_6\beta_4$  integrin (Xia et al., *J. Cell Biol.* 132:727-740, 1996). Indeed, this latter association is apparently essential for both hemidesmosome assembly, as well as the maintenance of the structural integrity of this cell matrix adhesion device (Baker et al., *J. Cell Sci.* 109:2509-2520, 1996; Jones et al. *Exp. Cell Res.* 213:1-11, 1994; Borradori et al., *Curr. Opin. Cell Biol.* 8:647-656, 1996). LN5/ $\alpha_6\beta_4$  complexes are believed to be conduits for signals from the external milieu of cells to the cytoplasm and potentially vice versa (Borradori et al., *Curr. Opin. Cell Biol.* 8:647-656, 1996; Mainiero et al., *EMBO J.* 16:2365-2375, 1997). In addition, it has been suggested that the interaction of LN5 with  $\alpha_6\beta_4$  integrins regulates the proliferation of keratinocytes and possibly other cells (Mainiero et al., *EMBO J.* 16:2365-2375, 1997). In particular, Mainiero et al. reported that an antibody that inhibits the function of the  $\beta_4$  subunits of  $\alpha_6\beta_4$  integrins inhibited proliferation of keratinocytes plated on LN5 (see the paragraph bridging pages 2371-1272 of Mainiero et al.). The interaction of LN5 with  $\alpha_3\beta_1$  integrins was reported to have no effect on the proliferation of cells.

The patents described above all relate to the ability of LN5 to promote epithelial and islet cell adhesion and proliferation. However, there is also a need for methods for inhibiting cell proliferation. The present invention addresses this need.

#### SUMMARY OF THE INVENTION

In one embodiment, the invention provides a method of interfering with cell proliferation comprising contacting a cell with a molecule which binds to laminin 5 and inhibits its function. Preferably, the molecule is a peptide. Preferably, the peptide is an integrin-derived peptide which comprises the binding site for laminin 5. More preferably the peptide is an antibody. Preferably, the antibody is specific for the  $\alpha_3$  subunit of laminin 5. More preferably, the antibody is specific for the G domain of the  $\alpha_3$  subunit of laminin 5.

In another embodiment, the invention provides a method of interfering with cell proliferation comprising contacting a cell comprising  $\alpha_3\beta_1$  integrins with a molecule that binds to the  $\alpha_3\beta_1$  integrins and inhibits their function. Preferably, the molecule is a peptide. Preferably, the peptide is a laminin 5-derived peptide. More preferably, the peptide comprises the G domain of the  $\alpha_3$  subunit of laminin 5. Alternatively, the peptide is preferably an antibody. Preferably, the antibody is specific for the  $\alpha_3$  subunits of  $\alpha_3\beta_1$  integrins. The finding that cell proliferation could be inhibited by a molecule that binds to  $\alpha_3\beta_1$  integrins and inhibits their function was quite surprising in view of the teaching of Mainiero et al., *EMBO J.* 16:2365-2375, 1997), discussed above, that  $\alpha_3\beta_1$  integrins were not involved in regulating cell proliferation.

In either of these embodiments, the cell may be contacted with the molecule *in vitro* or *in vivo*. The cell is contacted with the molecule *in vivo* by administering the molecule to a mammal in which the cell is located. Preferably, the cell is a tumor cell.

The invention also provides pharmaceutical compositions. These compositions comprise a molecule of the invention and a pharmaceutically-acceptable carrier.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing inhibition of 804G cell proliferation. 804G cells ( $2 \times 10^4$  cells/well of a 24-well tissue culture dish) were plated into complete serum-containing medium containing either 50  $\mu\text{g/ml}$  of CM6 LN5 function-inhibiting antibody or 5C5 control antibody. Every 24 hours, cells from three wells were trypsinized and counted. Every 48 hours, cells were given fresh medium containing the same concentration of antibody (indicated by asterisks).

Figure 2 is a graph showing the inhibition of proliferation of 804G cells plated on various substrates in the presence of CM6 monoclonal antibody. 804G cells were plated into culture wells ( $2 \times 10^4$  cells/well) of a 24-well tissue culture dish coated with 50  $\mu\text{g/ml}$  rat tail collagen (RTC), 25  $\mu\text{g/ml}$  fibronectin (FN), 25  $\mu\text{g/ml}$  laminin 1 (LN1) and 1  $\mu\text{g/ml}$  human laminin 5 (hLN5). Cells were maintained in medium supplemented with either 50  $\mu\text{g/ml}$  IgG control antibody or 50  $\mu\text{g/ml}$  LN5 function-inhibiting antibody CM6. At 48 hours, cells were trypsinized and counted. The "% proliferation" indicates the increase



in cell number as a percentage of that observed in the untreated control cell population. At 48 hours, the control cell population expanded from  $2 \times 10^4$  to  $1.09 \times 10^5$  cells (100%).

Figure 3A is a graph showing the inhibition of proliferation of MCF-10A cells plated onto surfaces coated with various extracellular matrix proteins in the presence of RG13 LN5  $\alpha 3$  subunit function-inhibitory antibody. MCF-10A cells ( $2 \times 10^4$  cells/well of a 24-well plate) were plated onto tissue culture plastic or onto surfaces coated with 50  $\mu\text{g/ml}$  RTC, 25  $\mu\text{g/ml}$  FN, 25  $\mu\text{g/ml}$  LN1 and 2  $\mu\text{g/ml}$  rat laminin 5 (rLN5). MCF-10A cells were maintained in medium supplemented with either 50  $\mu\text{g/ml}$  IgG control antibody or 50  $\mu\text{g/ml}$  RG13. At 48 hours, the cells were trypsinized and counted. The "% proliferation" indicates the increase in cell number as a percentage of that observed in the IgG-treated control cell population. The standard deviation was determined from the data derived from three trials. At 48 hours, the control cell population expanded from  $2 \times 10^4$  to  $1 \times 10^5$  cells (100%).

Figure 3B is a graph showing the inhibition of MCF-10A cell proliferation by the LN5 function-inhibiting antibody P3H9-2. MCF-10A cells ( $2 \times 10^4$  cells/well of a 24-well plate) were maintained in medium supplemented with either 50  $\mu\text{g/ml}$  IgG control antibody or 50  $\mu\text{g/ml}$  P3H9-2. At 48 hours, the cells were trypsinized and counted. The "% proliferation" indicates the increase in cell number as a percentage of that observed in the IgG-treated control cell population. The standard deviation was determined from the data derived from three trials.

Figure 3C is a graph showing the inhibition of MCF-10A cell proliferation by the LN5 function-inhibiting antibody BM165. MCF-10A cells ( $2 \times 10^4$  cells/well of a 24-well plate) were maintained in medium supplemented with either 50  $\mu\text{g/ml}$  IgG control antibody or 50  $\mu\text{g/ml}$  LN5 function-inhibiting antibody BM165. At 48 hours, the cells were trypsinized and counted. The "% proliferation" indicates the increase in cell number as a percentage of that observed in the IgG-treated control cell population. The standard deviation was determined from the data derived from three trials.

Figures 4A-4B are graphs showing the involvement of integrins in the proliferation of MCF-10A cells. In Figure 4A, MCF-10A cells were plated onto tissue culture plastic for 48 hours in the presence of 50  $\mu\text{g/ml}$  IgG control antibody, 25  $\mu\text{g/ml}$  of P1B5 (an  $\alpha_3$  integrin-inhibitory antibody), 25  $\mu\text{g/ml}$  of GoH3 (an  $\alpha_6$  integrin-inhibitory antibody), a

1:250 dilution of an inhibitory antibody against FN, and a 1:50 dilution of P1E6 (an  $\alpha_2$  integrin-inhibitory antibody). In Figure 4B, MCF-10A cells were plated into medium containing 50  $\mu$ g/ml IgG control antibody, 50  $\mu$ g/ml of RG13 antibody alone or together with a 1:5 dilution of hybridoma medium containing the  $\beta_1$  integrin-activating antibody TS2/16.2.1 or with 50  $\mu$ g/ml of the  $\beta_4$  integrin-activating antibody 3E1. For Figures 4A-B, at 48 hours, the cells were trypsinized and counted. The "% proliferation" indicates the increase in cell number as a percentage of that observed in the IgG-treated control cell population. The standard deviation was determined from the data derived from three trials. The control cell populations expanded from  $2 \times 10^4$  to  $1.18 \times 10^5$  cells (100%).

Figures 5A-C are graphs showing MAP kinase activity in 804G cells (Fig. 5A) and MCF-10A cells (Figs 5B-C) plated under various conditions. Cells were plated onto tissue culture plastic or surfaces coated with 50  $\mu$ g/ml RTC, 25  $\mu$ g/ml FN, 25  $\mu$ g/ml LN1 and 1  $\mu$ g/ml hLN5. 804G cells were maintained in medium supplemented with either 50  $\mu$ g/ml IgG control antibody or 50  $\mu$ g/ml CM6 antibody. After 48 hours, cells were scraped into gel sample buffer, processed for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and immunoblotted with either anti-ACTIVE MAPK p42/p44 to determine phosphorylated p42/44 or a probe for total p42/p44. Scan analyses of MAPK blots using the Bio-Rad Molecular Analyst program of 804G cells (Fig. 5A) and MCF-10A cells (Figs. 5B-C) are shown. The "amount" of total p42/p44 was normalized to that observed in IgG control-treated specimens, and then the levels of activated p42/p44 were appropriately adjusted. The % phosphorylated p42/p44 for each specimen relative to that observed in the IgG control samples was then calculated. The culture conditions and concentrations of antibodies used were identical to those given for the previous figures.

Figures 6A-6B are graphs showing the inhibition of proliferation for 804G (Fig. 6A) and MCF-10A (Fig. 6B) cells in the presence of inhibitory antibodies and the MAP kinase inhibitor PD98059. 804G cells (Fig. 6A) and MCF-10A cells (Fig. 6B) were maintained in medium containing 50  $\mu$ g/ml control IgG as well as 50  $\mu$ g/ml of either CM6 or RG13 antibodies. In some studies, the treated cells were plated onto substrate coated with 50  $\mu$ g/ml RTC or 1  $\mu$ g/ml of either hLN5 or rLN5. The MAPK inhibitor PD98059 in dimethylsulfoxide (DMSO) was added to cells at a concentration of 50  $\mu$ M. An equal

amount of DMSO lacking PD98059 was used as a control as indicated. At 48 hours, the cells were trypsinized and counted. The % proliferation was determined by evaluating the increase in cell number as a percentage of that observed in the IgG control cell population. The standard deviation was determined from the data derived from three trials. The control cell population expanded from  $2 \times 10^4$  to  $1.08 \times 10^5$  cells (100%) in Fig. 6A and from  $2 \times 10^4$  to  $1.05 \times 10^5$  cells (100%) in Fig. 6B.

#### DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The present invention includes the observations that molecules which bind to LN5 or to  $\alpha_3\beta_1$  integrins can interfere with cell proliferation. As used herein, the term "interfere" is meant to encompass both partial and complete inhibition of cell proliferation. Although the inhibition of epithelial cell proliferation is exemplified herein, the inhibition of any cell type dependent on LN5 for proliferation, or which expresses  $\alpha_3\beta_1$  integrins, is within the scope of the present invention.

The molecules interfere with cell proliferation by binding to LN5 or  $\alpha_3\beta_1$  integrins so as to inhibit their function. As used herein, "inhibiting the function" of LN5 means that the ability of LN5 to interact with  $\alpha_3\beta_1$  integrins and other integrins or cell receptors so as to provide a signal for cell proliferation is reduced or prevented. As described in the examples below, the function of LN5 produced by two different cell lines generated from two different organs of two different, distantly-related species was inhibited. In both instances, the division of cells in which LN5 function was perturbed was significantly inhibited. As used herein, "inhibiting the function" of  $\alpha_3\beta_1$  integrins means that the ability of the  $\alpha_3\beta_1$  integrins to interact with LN5 so as to provide a signal for cell proliferation is reduced or prevented.

In a preferred embodiment, the molecule which binds to LN5 or to  $\alpha_3\beta_1$  integrins to interfere with cell proliferation is a peptide. As used herein, "peptide" means a compound consisting of two or more amino acids linked covalently through peptide bonds. "Peptides" include oligopeptides, polypeptides, proteins, subunits of proteins, fragments of proteins, and portions of proteins.

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The peptide may be an integrin-derived peptide that binds to, and inhibits the function of, LN5. Such a peptide can interfere with cell proliferation by binding to LN5 and preventing LN5 from binding to, or otherwise interacting with, integrins on cell surfaces. Preferably, the integrin-derived peptide is an  $\alpha_3\beta_1$  integrin-derived peptide. Alternatively, the integrin-derived peptide may be a peptide derived from any other integrin that binds LN5, including an  $\alpha_6\beta_4$  integrin.

The peptide may also be an LN5-derived peptide which binds to, and inhibits the function of,  $\alpha_3\beta_1$  integrins. Such a peptide can inhibit cell proliferation by competitively inhibiting the binding of LN5 to  $\alpha_3\beta_1$  integrins on cell surfaces. Preferably, such a peptide comprises the G domain of the  $\alpha_3$  subunit of LN5 (see Example 6).

Suitable integrin-derived and LN5-derived peptides can be identified by means known in the art. For instance, they can be identified as described in the Examples below using function-inhibiting antibodies. They could also be identified by using fragments or portions of integrins or LN5 and testing for inhibition of cell proliferation. Since the sequences of the subunits of LN5 (GenBank L34155 ( $\alpha_3$ ), NM00228 ( $\beta_3$ ), and 215008 ( $\gamma_3$ )),  $\alpha_3\beta_1$  integrin (GenBank NM005501 ( $\alpha_3$ ) and X68969 ( $\beta_1$ )), and  $\alpha_6\beta_4$  integrin (GenBank X53586 ( $\alpha_6$ ) and X53587 ( $\beta_4$ )) are known, peptides useful in the practice of the invention can readily be prepared (*e.g.*, as described in Example 3 or by standard solid phase synthesis).

In a particularly preferred embodiment, the molecule that binds to LN5 or to  $\alpha_3\beta_1$  integrins to interfere with cell proliferation is an antibody. Antibodies suitable for use in the invention are known (see, *e.g.*, the Examples). The antibody can be directed against the  $\alpha_3$ ,  $\beta_3$  or  $\gamma_2$  chains of LN5. In a preferred embodiment, the antibody is directed against the  $\alpha_3$  subunit of LN5, more preferably against the G domain of the  $\alpha_3$  subunit. Alternatively, the antibody can be directed against the  $\alpha_3$  or  $\beta_1$  subunits of  $\alpha_3\beta_1$  integrins. In another preferred embodiment, the antibody is directed against the  $\alpha_3$  subunits of  $\alpha_3\beta_1$  integrins.

The antibody can be a polyclonal or monoclonal antibody. The term "antibody" also includes antibody fragments and derivatives, such as an  $F(ab')_2$  fragment, a Fab fragment, a monovalent antigen-binding fragment of an antibody composed of the variable regions from the heavy and light chains (Fv fragment), a single chain Fv fragment (scFv)

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with  $V_H$  and  $V_L$  joined by a polypeptide linker, a humanized antibody, a single chain antibody, other genetically engineered antibodies, or any other specific-binding antibody fragment or analog. An Fab fragment is an antibody fragment consisting of the entire light chain and the amino-terminal half of the heavy chain. A  $F(ab')_2$  chain is defined as an antibody fragment formed by cleavage of the constant region, resulting in two antigen combining sites linked by a disulfide bond. Methods of making antibodies, including antibody fragments and engineered antibodies, are, of course, well known. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1988) and Klein, *Immunology: The Science Of Self-Nonself Discrimination* (Wiley & Sons, Inc., New York, 1982).

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The ability of any molecule to inhibit LN5-mediated cell proliferation can be determined by a person of ordinary skill in the art using the assays described herein. Further, the ability of any molecule to bind to LN5 or to an  $\alpha_5\beta_1$  integrin can be easily determined using methods well known in the art, such as enzyme linked immunosorbent assay (ELISA) or immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

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To interfere with the proliferation of a cell, the cell is contacted with an effective amount of a molecule of the invention. The contacting may take place *in vitro* or *in vivo*. Methods and materials for *in vitro* cell cultures are well known in the art (see the Examples below), and effective amounts of a molecule of the invention for use in such cultures can be determined empirically, and doing so is within the skill in the art.

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To contact a cell *in vivo* with a molecule of the invention, the molecule is administered to an animal in which the cell is located. Preferably, the animal is a mammal, such as a cow, dog, cat, horse or human. Effective dosage forms, modes of administration and dosage amounts for the various molecules of the invention may be determined empirically, and making such determinations is within the skill in the art. It is understood by those skilled in the art that the dosage amount will vary with the particular molecule employed, the disease or condition to be treated, the severity of the disease or condition, the route(s) of administration, the rate of excretion of the molecule, the duration of the treatment, the identity of any other drugs being administered to the animal, the age, size and species of the animal, and like factors known in the medical and veterinary arts. In

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general, a suitable daily dose of a molecule of the present invention will be that amount of the molecule which is the lowest dose effective to produce a therapeutic effect. However, the daily dosage will be determined by an attending physician or veterinarian within the scope of sound medical judgment. If desired, the effective daily dose may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day. Administration of the molecule should be continued until an acceptable response is achieved.

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The molecules of the invention may be administered to an animal patient for therapy by any suitable route of administration, including orally, nasally, rectally, vaginally, parenterally (e.g., intravenously, intraspinally, intraperitoneally, subcutaneously, or intramuscularly), intracisternally, transdermally, intracranially, intracerebrally, and topically (including buccally and sublingually).

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While it is possible for a molecule of the invention to be administered alone, it is preferable to administer the molecule as a pharmaceutical formulation (composition). The pharmaceutical compositions of the invention comprise a molecule(s) of the invention as an active ingredient in admixture with one or more pharmaceutically-acceptable carriers and, optionally, with one or more other compounds, drugs, or other materials. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the animal. Pharmaceutically-acceptable carriers are well known in the art. Regardless of the route of administration selected, molecules of the present invention are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art. See, e.g., *Remington's Pharmaceutical Sciences*.

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LN5 is expressed at the leading edge of migrating tumor populations which may enhance cell proliferation and drive tumor growth. The method of the present invention can be used to inhibit tumor growth by interfering with LN5 function. Molecules which either bind to LN5 or bind to an  $\alpha_5\beta_1$  integrin, in a pharmaceutically acceptable carrier, are administered systemically, topically, or in the vicinity of, or directly into, a tumor. Tumors which can be treated according to the invention include carcinomas, such as breast, bladder, cervical, colon, lung, prostate, and skin carcinomas. It is also expected

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that other tumors and other proliferative disorders can be treated according to the invention to interfere with cell proliferation.

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In a preferred embodiment, for inhibition of tumor growth in a mammal, preferably a human, a composition comprising a molecule which binds to LN5 and inhibits its function, or which binds to an  $\alpha_5\beta_1$  integrin and inhibits its function, is injected into the mammal in a pharmaceutically-acceptable carrier in an amount ranging from about 0.01  $\mu\text{g/kg}$  to about 1,000  $\mu\text{g/kg}$ . In a particularly preferred embodiment, the amount administered is between about 1  $\mu\text{g/kg}$  and about 100  $\mu\text{g/kg}$ . Either single or multiple dosages may be administered. The composition may also be directly injected into a tumor, or adjacent the tumor, typically in an amount ranging from about 0.01  $\mu\text{g}$  to about 10 mg, preferably between about 0.1  $\mu\text{g}$  and about 1 mg.

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Suitable carriers for the preparation of solutions include water, polyols, sucrose, and glucose. Suitable carriers for the preparation of injectable solutions include water, alcohols, polyols, glycerol and vegetable oils. Pharmaceutical compositions for injection comprise pharmaceutically-acceptable sterile aqueous or non-aqueous liquids, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. These formulations can additionally contain preservatives, solubilizers, stabilizers, viscosity agents, wetting agents, emulsifiers, buffers, antioxidants and diluents.

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The compositions may advantageously be in a form suitable for topical administration, such as an ointment, foam, spray, gel, lotion, ointment, patch, drops, inhalant, cream, paste, suspension or dispersion.

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The compositions may also be provided as a controlled release composition for implantation at a site, *e.g.*, a site where inhibition of tumor growth is desired. For example, polylactic acid, polygalactic acid, regenerated collagen, poly-L-lysine, sodium alginate, gellan gum, chitosan, agarose, multilamellar liposomes and many other conventional depot formulations comprise bioerodible or biodegradable materials that can be formulated with biologically active compositions. These materials, when implanted or injected into or in the vicinity of a desired site (*e.g.*, into or near a tumor), gradually break down and release the active material to the surrounding tissue. For example, one method of encapsulating the compositions comprises the method disclosed in U.S. Patent No.

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4,391,909, the entire contents of which are hereby incorporated by reference. The use of bioerodible, biodegradable and other depot formulations is expressly contemplated in the present invention. Typically these compositions comprise the bioactive material combined with the carrier or depot material in a ratio of from about 1:1 to about 1:10,000, more preferably from about 1:10 to about 1:1,000. The use of infusion pumps and matrix entrapment systems for delivery of the compositions is also within the scope of the present invention. The compositions may also advantageously be enclosed in or combined with micelles or liposomes. Liposome encapsulation technology is well known in the art (*i.e.* Radin et al., *Meth. Enzymol.* 98:613-618, 1983).

The compositions can be packaged and administered in unit dosage form such as an injectable composition or local preparation in a dosage amount equivalent to the daily dosage administered to a patient or as a controlled release composition. A septum sealed vial containing a daily dose of the active ingredient in either PBS or in lyophilized form is an example of a unit dosage.

The compositions can also be used *in vitro* to assist in removal of cells from a tissue culture substrate. Typically, cell removal from a tissue culture plate is performed using an EDTA solution in combination with trypsin incubation. The inclusion of LNS function-inhibitory molecules will facilitate cell removal from tissue culture plates.



**EXAMPLES****Example 1: Cell cultures**

804G rat bladder carcinoma cells (ATCC CRL 11555) were maintained as described by Riddelle et al, *J. Cell Biol.* 112:159-168, 1991.

MCF-10A cells were obtained from the American Type Culture Collection (ATCC CRL 10317) (Rockville, MD) and maintained in a 1:1 mix of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 media supplemented with 5% equine serum, 0.01 mg/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin and 500 ng/ml hydrocortisone.

The TS2/16.2.1 mouse hybridoma line was obtained from the ATCC, and hybridoma supernatant containing TS2/16.2.1 antibody was collected from subconfluent dishes of actively growing cells (van de Wiel-van Kemenade et al., *J. Cell Biol.* 117:461-470, 1992).

OVCA429 cells were generously provided by Dr. Sharon Stack, Northwestern University Medical School, Chicago, IL. They were maintained according to Moser et al., *Int. J. Cancer*, 67: 695-701, 1996.

**Example 2: Anti-laminin 5 antibodies**

The BM165 antibody was provided by R. Burgeson, Harvard University, Cambridge, MA. It binds to human LN5 (hLN5) and inhibits its function

The CM6 antibody is species-specific and binds to the globular or G domain on the  $\alpha_3$  subunit of rat LN5 (rLN5) and inhibits rLN5 function. Baker et al., *J. Cell Sci.*, 109:2509-2530, 1996.

The control antibody 5C5 also binds to rLN5, but does not inhibit rLN5 function. Baker et al., *J. Cell Sci.*, 109:2509-2530, 1996.

The EM11 monoclonal antibody also binds to hLN5 and inhibits its function. This antibody was obtained from Desmos, Inc., San Diego, CA.

The P3H9-2 monoclonal antibody was obtained from Chemicon, Temecula, CA. It inhibits the function of hLN5.

RG13 mouse monoclonal antibody directed against the G domain of the  $\alpha_3$  subunit of hLN5 was prepared using MCF-10A LN5-containing matrix as immunogen as described in Langhofer et al., *J. Cell Sci.* 105:753-764, 1993.

Example 3: Preparation of recombinant G domain of LN5  $\alpha_3$  subunit

To produce the C-terminal G domain of hLN5  $\alpha_3$  subunit, a fragment encoding amino acid residues 747 to 1560 of the  $\alpha_3$  subunit was subcloned into the HindIII and Xho I sites of the pET32b vector (Novagen, Inc., Madison, WI) and transfected into DE3 $\alpha$  cells (Ryan et al., 1994, *J. Biol. Chem.*, **269**, 22779-22787). A His fusion protein was induced and the cells expressing the fusion protein were extracted in SDS buffer, as described in Example 8 below. The fusion protein of 110 kDa was identified using a His-HRP probe (SuperSignal HisProbe Western blotting kit, Pierce, Rockford, IL) and on an SDS-PAGE gel following protein staining in Coomassie Brilliant Blue (SIGMA, St. Louis, MO).

Example 4: Inhibition of 804G cell proliferation by CM6 antibody

Approximately  $2 \times 10^4$  804G cells were plated in medium containing CM6 antibody (see Example 2) and, at 24 hour intervals, cells were collected and counted (Fig. 1). The proliferation of CM6 antibody-treated cells was analyzed over a time period of 120 hours. As a control, 804G cells were treated with monoclonal antibody 5C5 (see Example 2) or mouse immunoglobulin G (IgG; purchased from Jackson ImmunoResearch, West Grove, PA). At 48 and 120 hours, the number of cells in CM6 antibody-treated cultures increased by only 37% and 56%, respectively, compared to control 5C5 antibody-treated cells (Fig. 1). 5C5 antibody had no obvious effect on 804G cell division when compared to the proliferation of untreated cells. Based on this analysis, 48 hours was selected as the time point for subsequent assays. Since IgG also had little effect on 804G cell proliferation (essentially the same as 5C5; data not shown), IgG-treated cells were used as the control population in all subsequent experiments.

It should be noted that the CM6 antibodies did not prevent 804G cells from attaching to, or in many instances, partially spreading onto their substrate. In addition, there was no significant detachment of cells during the course of the studies as determined

by counting any floating cells in the medium from the antibody-treated cell cultures each day. Furthermore, even though 804G cell growth was inhibited in CM6 antibody-containing medium, the treated cells were viable for up to ten days in the presence of antibody and could be induced to start to proliferate normally upon trypsinization and plating onto a fresh substrate in fresh medium. This indicates that the cells had not undergone terminal differentiation or apoptosis.

To confirm that CM6 antibodies impact 804G cell division, bromodeoxyuridine (BrdU) was used to label antibody-treated cells (Table 1). For the (BrdU) cell proliferation assays, cells were plated onto glass chamber slides under various conditions and, 18 hours later, 10  $\mu$ M BrdU (Sigma) was added directly to the cell culture medium. After 1 hour, the cells were extracted with methanol at -20°C and allowed to air dry. DNA was subsequently denatured by incubating the extracted cells in 2 N HCl at 37°C for 1 hour. The cell preparations were washed in PBS, then overlaid with a fluorescein-conjugated monoclonal anti-BrdU antibody (Boehringer-Mannheim, Indianapolis, IN) and incubated for 1 hour at 37°C. In this assay, only 12% of the CM6 antibody treated cells were stained compared to 44% of the control IgG treated 804G cells.

TABLE 1 - 804G cells

BrdU assay

	Cells Counted <sup>1</sup>	Labeling Index (%)
Control IgG	349	44.4
Anti-rat LN5 (CM6)	357	12.0

BM28 assay

	Cells Counted <sup>1</sup>	G1(%)	S(%)	G2(%)
Control IgG	371	43.8	33.7	22.4
Anti-rat LN5 (CM6)	210	44.3	38.5	17.2

<sup>1</sup>Total number of cells counted in three trials.

Example 5: CM6 antibody randomly blocks cell growth during the cell cycle

It was then determined, as described below, whether CM6 antibody blocks the growth of 804G cells at a particular stage in the cell cycle. For these studies, an antibody

against BM28/hMCM2 was used. BM28/hMCM2 is a member of the recently defined family of MCM proteins thought to play an essential role in the regulation of DNA replication (Kearsey et al., *Bioessays* 18:183-190, 1996). The BM28 protein, as well as other members of the MCM family, are found tightly bound to chromatin during the G1 phase of the cell cycle and are gradually released during S phase (Todorov et al., *J. Cell Biol.* 129:1433-1445, 1995; Krude et al., *J. Cell Sci.* 109:309-318, 1996). BM28 is detected using BM28 antibody following mild detergent extraction prior to cell fixation. The BM28 antibodies generate distinctive nuclear stains which are dependent upon the phases of the cell cycle (Todorov et al., *J. Cell Biol.* 129:1433-1445, 1995). For example, cell nuclei are stained uniformly bright in G1 cells, show a spotty pattern in S phase cells, and are practically unstained in G2 and in mitotic cells. Thus, BM28 staining, in combination with mild detergent extraction, provides a useful tool to visualize the cell cycle distribution pattern of a given cell population (Todorov et al., *J. Cell Biol.* 129:1433-1445, 1995).

For visualization of BM28 antigen, 804G cells grown on coverslips were washed in phosphate buffered saline (PBS) containing 2 mM MgCl<sub>2</sub> and extracted in 0.5% Triton X-100, 20 mM Tris HCl, pH 7.4, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 0.5 mM phenylmethyl sulfonyl fluoride for 5 minutes at 20°C. Cells were washed in PBS, fixed and extracted in methanol (-20°C), followed by acetone (-20°C), and processed for indirect immunofluorescence using affinity-purified rabbit anti-BM28 antibodies and a fluorescein-conjugated anti-rabbit immunoglobulin secondary antibody (Jackson ImmunoResearch, West Grove, PA) (Todorov et al., *J. Cell Sci.*, 107:253-265, 1994). DNA was visualized by staining with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Fixed and stained cells were viewed using a Zeiss (Thornwood, NY) Photomicroscope III fitted with epifluorescence optics.

The nuclei of both the CM6 and IgG treated cells showed the full range of BM28 staining patterns. Furthermore, the percentage of nuclei at different cell cycle stages as indicated by antibody staining was similar in 804G cell populations maintained in normal medium, in medium supplemented with CM6 antibody, and in medium containing control IgG antibody (Table 1). This suggests that CM6 antibody blocks cell growth randomly during the cell cycle.

To determine whether growth of CM6-treated 804G cells could be restored, cells were plated on defined extracellular matrix proteins including fibronectin (FN), laminin 1 (LN1), rat tail collagen (RTC) type I, and LN5. LN1, FN and RTC were obtained from Collaborative Research (Bedford, MA) and were coated onto cell supports according to the instructions of the supplier. rLN5 and hLN5 were prepared from 804G or MCF-10A cell conditioned medium, respectively (Baker et al., *J. Cell Sci.*, 109: 2509-2520, 1996; Stahl et al., *J. Cell Sci.*, 110:55-63, 1997). In brief, cell medium was fractionated by cation exchange chromatography. Fractions enriched in LN5 were further processed by anion exchange chromatography and a final purification was achieved using hydroxyapatite chromatography as described in U.S. Patent No. 5,760,179. For studies involving LN5, hLN5 was used which is not recognized by the rat specific CM6 antibodies. Inhibition of division of 804G cells treated with CM6 was not reversed by maintaining the cells on LN1 or FN, since the increase in cell number was only 32% and 6.2% of that observed in control antibody cell populations (Fig. 2). In contrast, proliferation of 804G cells which had been plated onto RTC-coated and hLN5-coated substrates and treated with CM6 antibody is 75.5% and 73.8%, respectively, of that of control antibody treated cells (Fig. 2).

Example 6: Antibodies against hLN5 inhibit proliferation of MCF-10A cells

To determine whether the inhibition of proliferation discussed above also occurs with other cell types and antibodies, comparable studies were performed using cells from a different species (human) as well as several different function-inhibiting LN5 antibodies, particularly P3H9-2, BM165 and RG13 (see Example 2). RG13 antibodies, like P3H9-2 and BM165 antibodies, inhibit rapid adhesion of epithelial cells to hLN5. The human cell line used for this study was MCF-10A which is derived from human breast epithelium and expresses LN5 *in vitro* (Stahl et al., *J. Cell Sci.*, 110:55-63, 1997; U.S. Patent No. 5,770,448). Indeed, immunochemical and molecular analyses of MCF-10A cells reveal that these cells secrete a matrix whose major component is LN5 (Stahl et al., *J. Cell Sci.*, 110:55-63, 1997). This matrix does not contain any detectable amounts of LN6 or LN7, both of which, like LN5, contain an  $\alpha_3$  subunit.

Antibodies RG13, P3H9-2 and BM165 all significantly reduced cell division of MCF-10A cells by 62%, 47.4% and 41.9%, respectively, compared to IgG-treated control cell populations (Figs 3A-C). They do so with little, if any, apparent effect on the spreading of the cells onto their substrate after 24 hours.

Only 39% of cells were stained with BrdU antibody in RG13 antibody-treated MCF-10A cultures compared to 52% of cells which were stained in MCF-10A cells treated with IgG control antibody (Table 2). MCF-10A cells treated with the other hLN5 inhibitory antibodies showed similar staining patterns. In addition, as observed with CM6 treated 804G cells, the hLN5 inhibitory antibodies apparently blocked MCF-10A cells randomly in the cell cycle as determined using the BM28 antibody marker (Table 2).

TABLE 2 - MCF-10A cells

BrdU assay

	Cells Counted <sup>1</sup>	Labeling Index (%)
Control IgG	302	52.0
Anti-hLN5 (RG13)	301	39.0

BM28 assay

	Cells Counted <sup>1</sup>	G1(%)	S(%)	G2(%)
Control IgG	456	43.2	34.0	22.8
Anti-hLN5 (RG13)	343	40.8	33.5	25.7

<sup>1</sup>Total number of cells counted in three trials.

The negative effect of RG13 antibodies on the growth of MCF-10A cells was overcome by maintaining the cells on RTC and rLN5, but not by plating the cells on LN1 or FN (Fig. 3A). MCF-10A cells maintained on RTC in the presence of RG13 antibodies showed a proliferation of 107.4% compared to IgG-treated control cells (Fig. 3A). When maintained on rLN5, the growth of MCF-10A cells in RG13 antibodies was 104% compared to IgG-treated control cells (Fig. 3A).

CM6 antibody treated 804G cells only partially flatten onto their substrate compared to their control counterparts, although, as noted above, they clearly attach. The function-inhibitory antibodies against hLN5 have no apparent effects on MCF-10A cellular

morphology. The cells appear to spread fully onto their substrate in the presence of the inhibitory antibodies. This indicates that the inhibition of cell division induced by inhibiting LN5 function in both 804G and MCF-10A cells is not a secondary consequence of the loss of cell-substrate contact which is known to trigger the differentiation of many epithelial cell types (Adams et al., *Nature*, 340:307-309, 1989). Rather, it is the result of a block in a signal "encoded" by LN5 which is transduced by means of cell surface receptors to the overlying cells and which can directly modulate the progress of the cell cycle.

The LN5 function-perturbing antibodies CM6 and RG13 recognize the  $\alpha_3$  subunit of the LN5 heterotrimer (Baker et al., *J. Cell Sci.*, 109:2509-2520, 1996). CM6 antibodies have been localized to the G domain of the intact LN5 molecule, while RG13 antibodies recognize the G domain of human  $\alpha_3$  prepared in a bacterial expression system. Thus, the proliferative impact of LN5  $\alpha_3$  subunit is encoded by a sequence of amino acids in or close to the G domain. This domain is likely to be the  $\alpha_3\beta_1$  binding site within the LN5 molecule.

#### Example 7: Antibodies against integrins inhibit proliferation of MCF-10A cells

The potential involvement of integrin receptors in the inhibition of cell proliferation was investigated using MCF-10A cells. LN5 has two known integrin receptors,  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$  (Carter et al., *J. Cell Biol.*, 111:3141-3154, 1990). Thus, antibodies P1B5 and GoH3 (obtained from Life Technologies, Gaithersburg, MD), which perturb  $\alpha_3$  and  $\alpha_6$  integrin function, respectively, were used. Antibody P1B5 inhibited proliferation of MCF-10A cells maintained on tissue culture plastic or RTC by 62.6% and 77.3%, respectively, compared to control IgG treated cells (Fig. 4A). In contrast, GoH3 inhibited MCF-10A cell proliferation by 30.2% (Fig. 4A). In control studies, antibodies which inhibit the function of FN (anti-FN antibody (clone II); Life Technologies, Gaithersburg, MD) and  $\alpha_2$  integrin (antibody P1E6; Life Technologies, Gaithersburg, MD) have no apparent effect on MCF-10A cell division. The latter result suggests that LN5 "signals" are not transduced via some sort of "crosstalk" between  $\alpha_3\beta_1$  and  $\alpha_2\beta_1$  integrin (Zhang et al., *Exp. Cell Res.* 227:309-333, 1996). It should also be noted that the proliferation of MCF-10A

cells which have been treated with P1B5 antibody for 48 hours, was restored to normal following plating into fresh medium in the absence of antibody.

To confirm that the anti-FN and P1E6 antibodies were function-inhibiting, adhesion assays, in which  $2 \times 10^5$  MCF-10A cells and OVCA429 cells were plated onto either FN-coated or RTC-coated, respectively, wells of a 96-well plate (Sarsedt, Newton, NC), were performed. After 30 minutes at 37°C, the cells were washed extensively in Dulbecco's PBS, fixed for 15 minutes in 3.7% formaldehyde in PBS, and then incubated at room temperature with 0.5% crystal violet for 10 minutes. The dye was then solubilized with 1% SDS (100  $\mu$ l/well), and absorbance at 570 nm measured on a Vmax plate reader (Molecular Devices, Menlo Park, CA). The anti-FN antibody (1:250 dilution) inhibited the attachment of MCF-10A cells to the FN-coated wells by 37%, and P1E6 antibody (1:50 dilution) inhibited the adhesion of OVCA429 cells to RTC by 49%.

The above results suggest that  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$  integrins mediate the proliferation effects of LN5 on MCF-10A cells. To provide further support for this possibility, antibodies TS2/16.2.1 (see Example 1) and 3E1 (Life Technologies, Gaithersburg, MD), which activate  $\beta_1$  and  $\beta_4$  integrins, respectively, in the absence of ligand (van de Wiele-van Kemenade et al., *J. Cell Biol.*, 117:461-470, 1992; Mainiero et al., *EMBO J.*, 16: 2365-2375, 1997) were used. MCF-10A cells were treated with a combination of RG13 and TS2/16.2.1 or RG13 and 3E1 antibodies. The proliferation of such antibody treated cells was 99% and 82%, respectively, of control antibody treated cell cultures (Fig. 4B). Thus, these antibodies rescue proliferation, and these results provide further evidence that  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$  integrins mediate the proliferation effects of LN5 on MCF-10A cells.

#### Example 8: Antibody-mediated inhibition of MAP kinase cascade

MAP kinase may play a role in mediating the regulatory effects of extracellular matrix and integrin receptors on cell cycle progression. The degree of MAP kinase activation in both 804G and MCF-10A cells was evaluated following various treatments (Rosales et al., *Biochim. Biophys. Acta.* 1242:77-98, 1995; Schlaepfer et al., *Trends Cell Biol.* 8:151-157, 1998; Howe et al., *Curr. Opin. Cell Biol.* 10:220-231, 1998). For these studies, an antibody (affinity-purified polyclonal anti-ACTIVE MAPK p42/p44; Promega, Madison, WI), which is specific for phosphorylated, activated p42/p44, and an antibody



which recognizes p42/p44 irrespective of its activation state (anti-p42/p44; Santa Cruz Biotechnology, Santa Cruz, CA) were used. Under each experimental condition, both total MAP kinase content and the level of activated MAP kinase in cell extracts were evaluated by SDS-PAGE and immunoblotting as previously described (Zachroff et al., *J. Cell Biol.*, 98:1231-1237, 1984; Kiatte et al., *J. Cell Biol.*, 109:3377-3390, 1989), except that blots were developed using a chemiluminescence kit (Pierce, Rockford, IL). Immunoblots were scanned and quantitated using Molecular Analyst (BioRad, Richmond, CA).

In 804G cells and MCF-10A cells treated with antibodies that inhibit the function of  $\alpha_3$  subunit of LN5, MAP kinase activity was reduced by 61.1 % and 44.2%, respectively, relative to MAP kinase activity in control IgG-treated cell populations (Figs. 5A-B). In contrast, in 804G cells maintained on RTC or hLN5 in the presence of CM6 antibodies, MAP kinase activity was at 189.5% and 187.1% of that in control IgG-treated cells (Fig. 5A). MAP kinase activity remained down-regulated in CM6-treated 804G cells plated onto LN1- and FN-coated substrates, the level being 33.1% and 52.8%, respectively, of that observed in control IgG-treated cells (Fig. 5A).

In MCF-10A cells maintained on RTC or rLN5 in the presence of RG13 antibodies, MAP kinase activity was 107.1% and 79.7% of that in control IgG-treated cells, respectively (Fig. 5B). MAP kinase activity in RG13-treated MCF-10A cells plated onto LN1 and FN-coated substrate was 43.1% and 130.3%, respectively, of that observed in control IgG-treated cells. (Fig. 5B).

MAP kinase activity was then analyzed in MCF-10A cells treated with integrin-blocking antibodies. MAP kinase activity was reduced by 37.1% and 30.7%, respectively, in MCF-10A cells treated with P1B5 or GoH3 antibodies compared to IgG-treated control cells (Fig 5C). TS2/16.2.1 antibodies restore MAP kinase activity in MCF-10A cells treated with RG13 antibodies to 95.1% of control levels (Fig. 5C).

These results reveal a correlation between MAP kinase activity and LN5 regulation of cell proliferation with the notable exception that FN is able to rescue MAP kinase activity in MCF-10A cells, but not the proliferation of MCF-10A cells treated with LN5 function-inhibitory antibodies. Thus, to provide additional evidence that MAP kinase is part of the pathway by which LN5 regulates cell proliferation, the MAP kinase inhibitor

PD98059 (New England Biolabs, Beverly, MA) was used. A stock solution of inhibitor at a concentration of 50 mM in dimethylsulfoxide (DMSO) was prepared. The inhibitor was added directly to complete medium to a final concentration of 50  $\mu$ M. The same volume of DMSO without inhibitor was added to cell cultures as a control. After 48 hours, cells were washed and harvested in Laemmli-type gel sample buffer containing 2% SDS. The cell extracts were sonicated briefly and heated at 95°C for 3 minutes before gel electrophoresis (Laemmli, *Nature*, 277:680-685, 1970). Cells treated with this inhibitor did not die during the course of these assays as determined by a trypan blue exclusion assay. MAP kinase is efficiently inactivated in 804G and MCF-10A cells treated with the inhibitor, as determined by immunoblotting using the anti-ACTIVE MAPK p42/p44 antibody probe. Furthermore, in all cases, PD98059 inhibited proliferation of both 804G and MCF-10A cells. This is true even for cells treated with LN5-perturbing antibodies and plated onto substrates that have been shown to "rescue" cell division (Figs. 6A-B).

The MAP kinase analyses suggest that this enzyme is a component of a pathway that transduces signals from LN5 via  $\alpha_5\beta_1$  integrin complex to the cell nucleus where they regulate cell division. This conclusion is based on the following: 1) decreased activity of MAP kinase in 804G and MCF-10A cells treated with LN5 function-inhibitory antibodies; 2) an inhibition of division of these same cell types when treated with a MAP kinase inhibitor; and 3) MAP kinase activity is restored close to, or greater than, normal levels in cells treated with LN5 antibodies when they are maintained on LN5 or RTC or when their  $\beta_1$  integrin is activated, under which conditions the proliferation of the cells is "rescued."

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

## Claims

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WHAT IS CLAIMED IS:

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1. A method of interfering with cell proliferation comprising contacting a cell with a molecule which binds to laminin 5 and inhibits its function.

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2. The method of Claim 1 wherein the molecule is contacted with the cell *in vivo* by administering the molecule to a mammal in which the cell is located.

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3. The method of Claim 2, wherein said molecule is a peptide.

4. The method of Claim 3, wherein said peptide is an integrin-derived peptide which comprises the binding site for laminin 5.

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5. The method of Claim 3, wherein said peptide is an antibody.

6. The method of Claim 5, wherein said antibody is selected from the group consisting of a humanized antibody, a single chain antibody, a genetically engineered antibody, a F(ab')<sub>2</sub> fragment, a Fab fragment, an Fv fragment and a single chain Fv fragment.

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7. The method of Claim 5, wherein said antibody is specific for the  $\alpha_3$  subunit of laminin 5.

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8. The method of Claim 7, wherein said antibody is specific for the G domain of the  $\alpha_3$  subunit of laminin 5.

9. The method of Claim 5, wherein said antibody is selected from the group consisting of CM6, RG13, P3H9-2, and BM165.

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10. The method of Claim 2 wherein said cell is a tumor cell.

11. The method of Claim 10 wherein said tumor is a carcinoma.

12. A pharmaceutical composition comprising a pharmaceutically-acceptable carrier and a molecule which binds to laminin 5 and inhibits its function.

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13. The composition of Claim 12, wherein said molecule is a peptide.

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14. The composition of Claim 13, wherein said peptide is an integrin-derived peptide which comprises the binding site for laminin 5.

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15. The composition of Claim 13, wherein said peptide is an antibody.

16. The composition of Claim 15, wherein said antibody is specific for the  $\alpha_3$  subunit of laminin 5.

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17. The composition of Claim 16, wherein said antibody is specific for the G domain of the  $\alpha_3$  subunit of laminin 5.

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18. The composition of Claim 15, wherein said antibody is selected from the group consisting of CM6, RG13, P3H9-2, and BM165.

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19. A method of interfering with cell proliferation comprising contacting a cell comprising  $\alpha_3\beta_1$  integrins with a molecule which binds to an  $\alpha_3\beta_1$  integrin and inhibits its function.

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20. The method of Claim 19 wherein the molecule is contacted with the cell *in vivo* by administering the molecule to a mammal in which the cell is located.

21. The method of Claim 20, wherein said molecule is a peptide.

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22. The method of Claim 21, wherein said peptide is a laminin 5-derived peptide.

23. The method of Claim 22, wherein the peptide comprises the G domain of the  $\alpha_3$  subunit of laminin 5.

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24. The method of Claim 21, wherein said peptide is an antibody.

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25. The method of Claim 24, wherein said antibody is selected from the group consisting of a humanized antibody, a single chain antibody, a genetically engineered antibody, a F(ab')<sub>2</sub> fragment, a Fab fragment, an Fv fragment and a single chain Fv fragment.

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26. The method of Claim 24, wherein said antibody is specific for the  $\alpha_3$  subunit of the  $\alpha_3\beta_1$  integrin.

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27. The method of Claim 24, wherein said antibody is P1B5.

28. The method of Claim 20 wherein said cell is a tumor cell.

29. The method of Claim 28 wherein said tumor is a carcinoma.

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30. A pharmaceutical composition comprising a pharmaceutically-acceptable carrier and a molecule which binds to an  $\alpha_3\beta_1$  integrin and inhibits its function.

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31. The composition of Claim 30, wherein said molecule is a peptide.

32. The method of Claim 31, wherein said peptide is a laminin 5-derived peptide.

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33. The composition of Claim 32, wherein the peptide comprises the G domain of the  $\alpha_3$  subunit of laminin 5.

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34. The composition of Claim 31, wherein said peptide is an antibody.

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35. The composition of Claim 34, wherein the antibody is specific for the  $\alpha$ ,  
subunit of the  $\alpha_3\beta_1$  integrin.

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36. The composition of Claim 34, wherein said antibody is P1B5.

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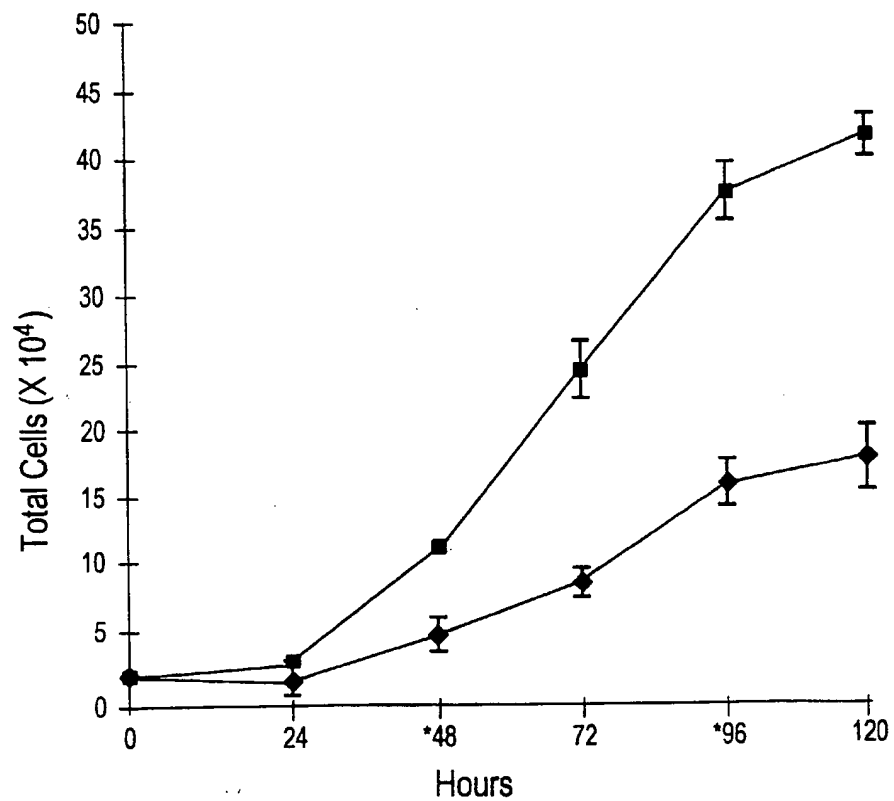


FIG. 1

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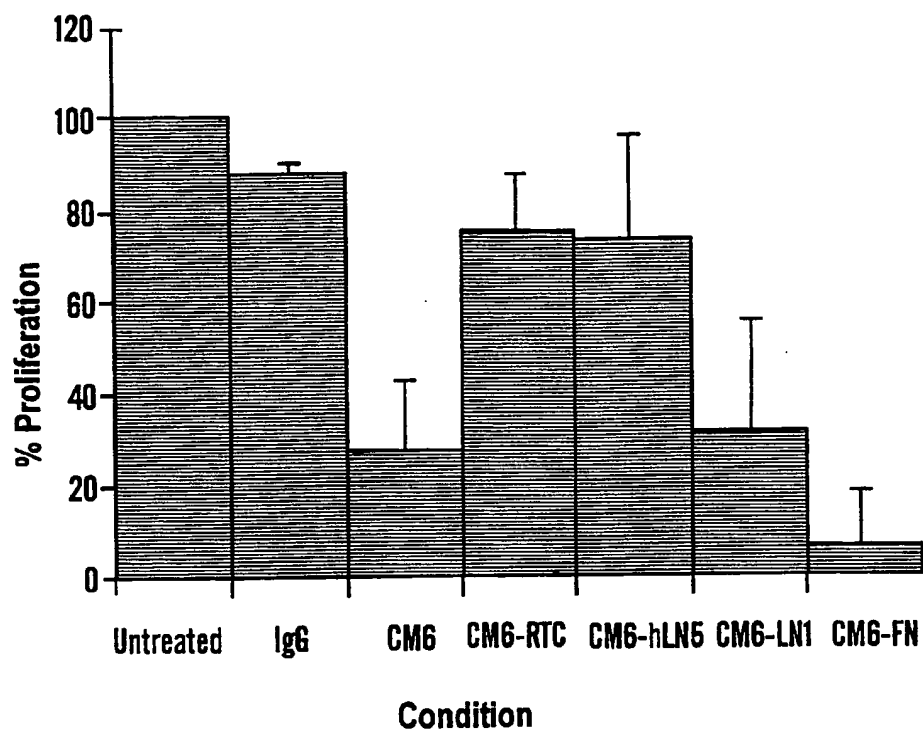
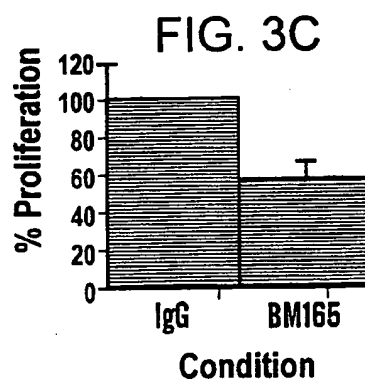
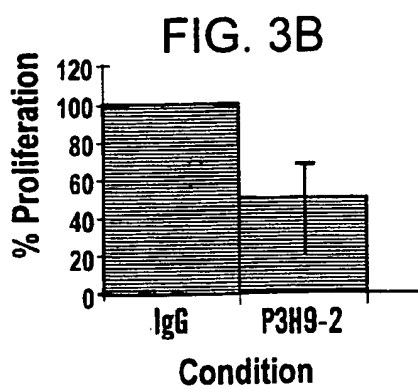
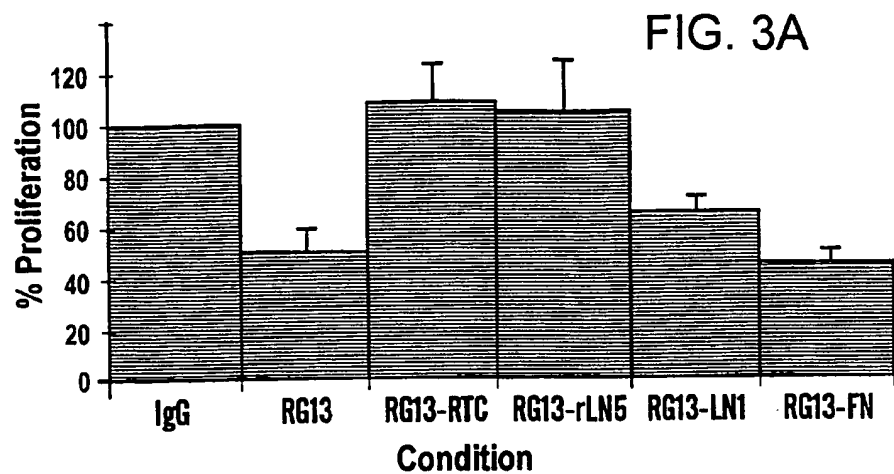


FIG. 2



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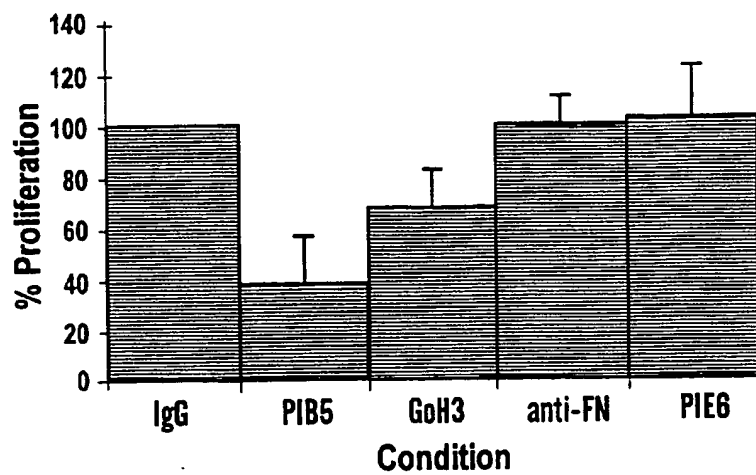


FIG. 4A

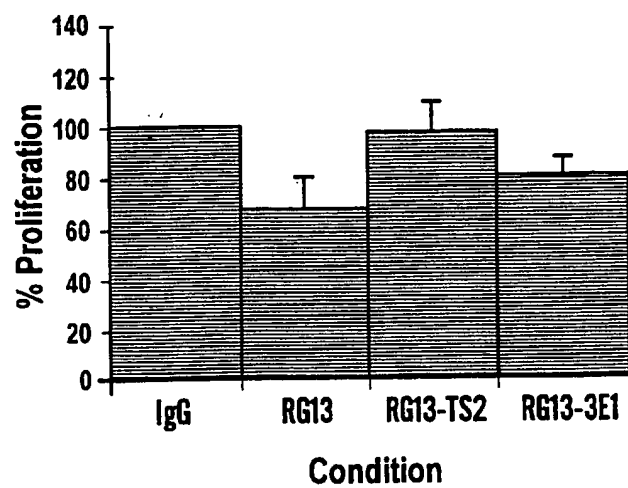
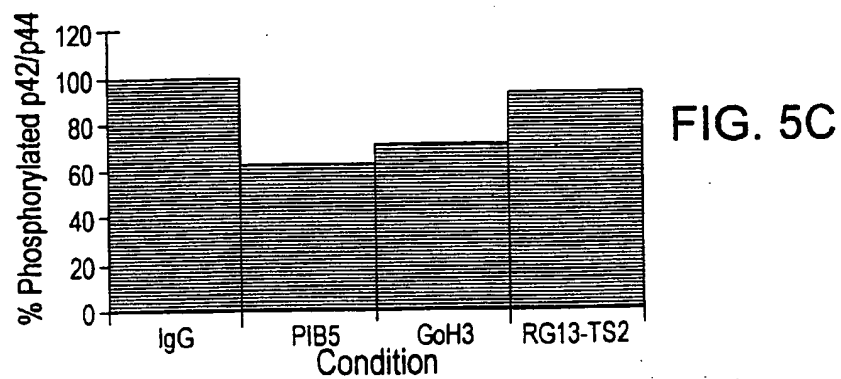
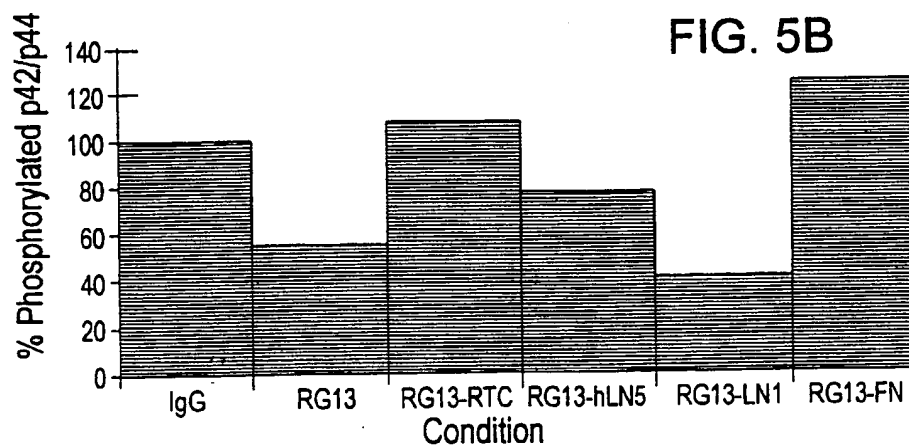
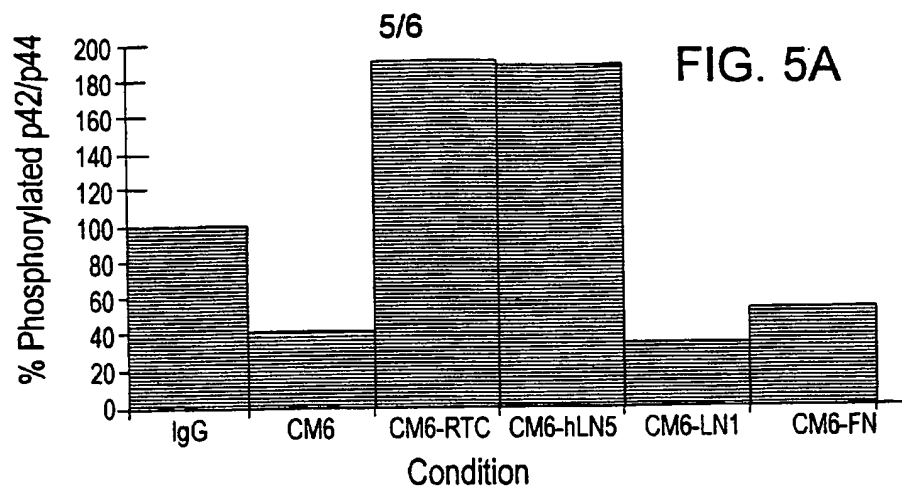


FIG. 4B



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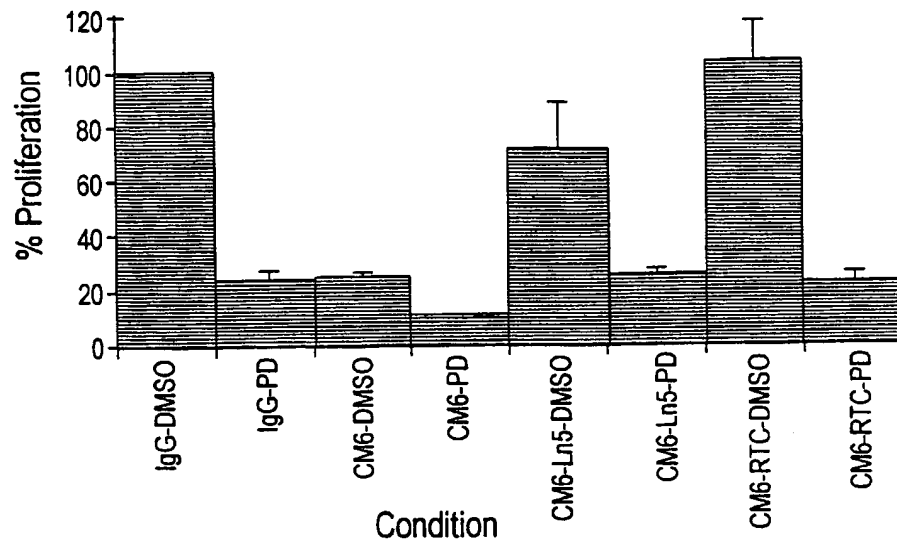


FIG. 6A

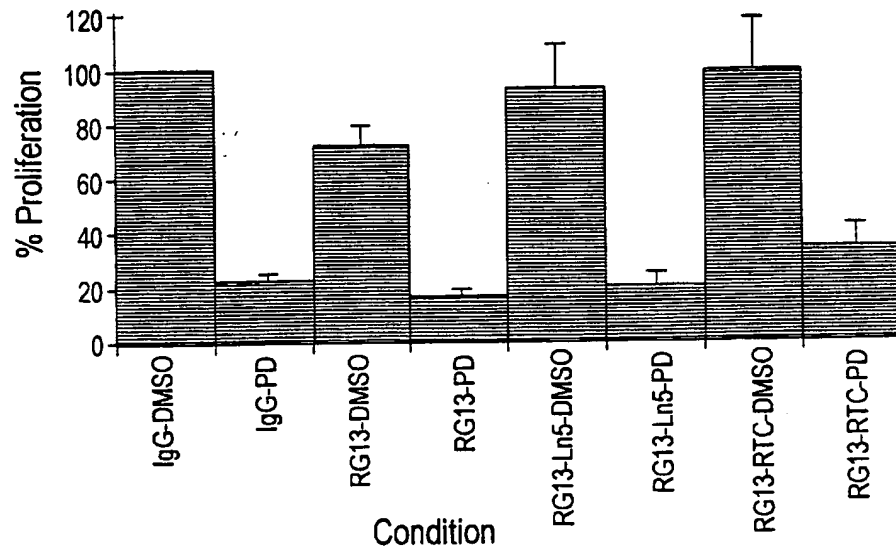


FIG. 6B

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/25359

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 5/02; A61K 39/00; A01N 37/18; A61K 39/395  
US CL : 435/325, 424/277.1, 514/2, 424/135.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 424/277.1, 514/2, 424/135.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN. WEST

laminin 5 alpha 3 subunit g domain, anchoring filament protein, kalinin/nicein.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCMILLIAN et.al., Immunohistochemical analysis of the skin in junctional epidermolysis bullosa using laminin 5 chain specific antibodies is of limited value in predicting the underlying gene mutation. British Journal of Dermatology. 1997, Vol. 136. pages 817-822, especially pages 818,821	9
Y	DIPERSIO, C. M. et al. alpha3beta1 integrin is required for normal development of the epidermal basement membrane. The Journal of Cell Biology. 05 MAY 1997. Vol. 137. No. 3. pages 729-742.	9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

\* Special categories of cited documents:

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\*B\* earlier document published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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\*A\* document member of the same patent family

Date of the actual completion of the international search

21 JANUARY 2000

Date of mailing of the international search report

28 FEB 2000

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